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Methods in Ecology and Evolution

REVIEW

Sampling mass mortality events to enable diagnoses: A protocol using freshwater mussels

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Abstract

1. Many taxa around the globe are threatened by often unexplained mass mortality events (MMEs), which can decimate populations and compromise key ecosystem functions. One example of a highly threatened taxon facing frequent MMEs is freshwater mussels (Unionida).
2. There has been a recent increase in interest in understanding the causes of freshwater mussel MMEs, but standardised methodologies for how best to respond to them to facilitate diagnoses are unavailable. When an MME is observed, swift and appropriate sample collection is imperative owing to the transient nature of these phenomena.
3. Here we provide structured guidance that will facilitate rapid and appropriate sampling of MMEs, using freshwater mussels as an example. We set out standardised procedures for sample collection, preparation and preservation.
4. The procedures we outline will improve our capacity for diagnostic investigations of MMEs and other mortality events, not only in freshwater mussels but also across many other taxa. This, in turn, can inform appropriate management responses.

KEYWORDS
die-off, diseases, freshwater mussel, histology, mortality event, protocol, sampling methods, Unionida

1 | INTRODUCTION

Mass mortality events (MMEs) are rapidly occurring demographic catastrophes involving mortality rates higher than background levels (Fey et al., 2015). MMEs can alter food web structures and change community composition (Baruzzi et al., 2018; Fey et al., 2019), disrupt ecosystem function (Fey et al., 2019) and influence the survival of species (García-March et al., 2020). Recently reported MMEs include the death of hundreds of South American sea lions (*Otaria byronia*) in Argentina due to an outbreak of highly pathogenic avian influenza A (Rimondi et al., 2024), tsunami-induced burial and starvation of the long-lived and sparsely populated clam *Mercenaria stimpsoni* (Kubota et al., 2021), and the loss of over 200,000 Saiga antelope (*Saiga tatarica*) in 3 weeks in central Kazakhstan due to bacterial-induced (*Pasteurella multocida*) haemorrhagic septicaemia (Kock et al., 2018). Unlike these cases, there have been many MMEs for which the cause remains undetermined (Fey et al., 2015; Hamilton et al., 2021; Richard et al., 2021; Waller & Cope, 2019; Young, 1994). This is partly due to the challenges in implementing a timely and comprehensive diagnostic investigation. Given that the frequency and magnitude of reported MMEs have increased in many phyla in recent decades (Fey et al., 2015), standardised mortality response and sampling methods could help overcome these challenges and lead to a better understanding of the causes of MMEs.

Freshwater mussels (Unionida) are among the most highly threatened faunal groups worldwide (Lopes-Lima et al., 2018). Since the 1940s and 1950s, MMEs have been reported in freshwater mussel populations (Downing et al., 2010; Neves, 1987; Waller & Cope, 2019), with recent examples including the MMEs of pheasant-shell (*Ortmanniana pectorosa*) in the Clinch River, VA and TN, USA, during autumn each year between 2016 and 2019 (Leis et al., 2019; Richard, 2018; Richard et al., 2020, 2021), of mucket (*Ortmanniana ligamentina*) in the Huron River, MI, USA, in September 2018 (Richard et al., 2022), of multiple Unionida species in the Odra River, Poland, in the summer of 2022 (Marchowski et al., 2024; Sobieraj & Metelski, 2023; Szlauer-Łukaszewska et al., 2024), and of freshwater pearl mussels (*Margaritifera margaritifera*) in rivers across Sweden between 2005 and 2020 (Alfjorden et al., 2024; Wengström et al., 2019).

Considering that freshwater mussels maintain water quality, increase habitat complexity, and influence nutrient cycles, among other ecosystem functions (Zieritz et al., 2022), mussel MMEs could cause flow-on effects in the whole ecosystem (DuBose et al., 2019; Vaughn et al., 2015). Despite this, many freshwater mussel MMEs are not systemically investigated, in part due to a lack of responders and clear sampling protocols for MMEs.

The overall objective of an MME investigation is to identify potential causative factors that may have led to the event (Work, 2015). To investigate MMEs, the steps used during a diagnostic investigation to identify the cause of death in a single animal are applied on a larger scale. This involves a combination of standardised field observations and diagnostic tests (Figure 1). A central component of the diagnostic investigation is the postmortem examination (that is, necropsy or autopsy on euthanised moribund (sick) or dead individuals), where tissues are examined at the gross ('naked eye') and microscopic level, along with the collection of samples for other diagnostic tests. The postmortem exam allows the identification of pathological processes and indicates potential causative agents for future research. Correlations between pathological findings and diagnostic test results subsequently help evaluate potential causes. Any diagnostic investigation depends on those working in a field setting collecting the right information and samples needed to execute these steps.

Although information is available for tips regarding the process of conducting an MME investigation (e.g. Abila et al., 2018; Meyer & Barclay, 1990; Southwick & Loftus, 2017; Work, 2015), preserving samples from marine mollusc MMEs (e.g. EURL for Molluscs Diseases, 2020), and quantifying MMEs (Marchowski et al., 2024), a detailed and widely available protocol for how to collect and preserve the samples needed to undertake an MME diagnostic investigation is lacking, especially for freshwater mussels.

In this paper, we provide a protocol for sampling an MMEs in a manner that will facilitate an MME investigation through the application of a wide range of diagnostic methods. These methods can be used to investigate the role of many potential causes previously implicated in MMEs of both freshwater and marine bivalves, where the cause is not immediately obvious, including microbes (Alfjorden et al., 2024; Da Silva Neto et al., 2024; Leis, Dziki, Richard, et al., 2023; Richard et al., 2020, 2021; Zhong et al., 2016), macroparasites (Jonsson & Andé, 1992; Thieltges, 2006), transmissible cancer (Carballal et al., 2015; Charles et al., 2020; McGladdery et al., 2001), harmful chemicals (e.g. heavy metals, organic pollutants, or algal or cyanobacterial bloom produced biotoxins) (Donaghy et al., 2016; Fleming et al., 1995; Neves, 1987; Sobieraj & Metelski, 2023), and various environmental factors such as low oxygen or high temperatures (Neves, 1987; Soon & Ransangan, 2019; Werner & Rothhaupt, 2008).

Methods involved in investigating potential causes of mussel losses that are obvious or associated with longer-term declines are not addressed in this paper. Examples of such causes include drought (Atkinson et al., 2014; DuBose et al., 2019; McDowell & Sousa, 2019), flooding (Downing et al., 2010; Haag, 2012; Sousa et al., 2012), impoundment (Tiemann et al., 2007; Vaughn & Taylor, 1999; Watters, 1996; Watters & Flaute, 2010), predation (Downing et al., 2010; Strayer et al., 2004), physical habitat destruction (Downing et al., 2010; Haag, 2012; Strayer et al., 2004), and reductions in host fish abundance (Downing et al., 2010; Haag, 2012; Strayer et al., 2004; Watters, 1996).

This paper aims to provide a protocol that equips non-specialist first responders with a core procedure that will allow a rapid response to MMEs (Figure 1), with a focus on sample collection and preservation. Using our procedures will enable a subsequent diagnostic investigation with the support of collaborations established before or after sampling. Although the protocol is developed for MMEs in freshwater mussel populations, the approach can be readily adapted for many taxa across a variety of scales and settings.

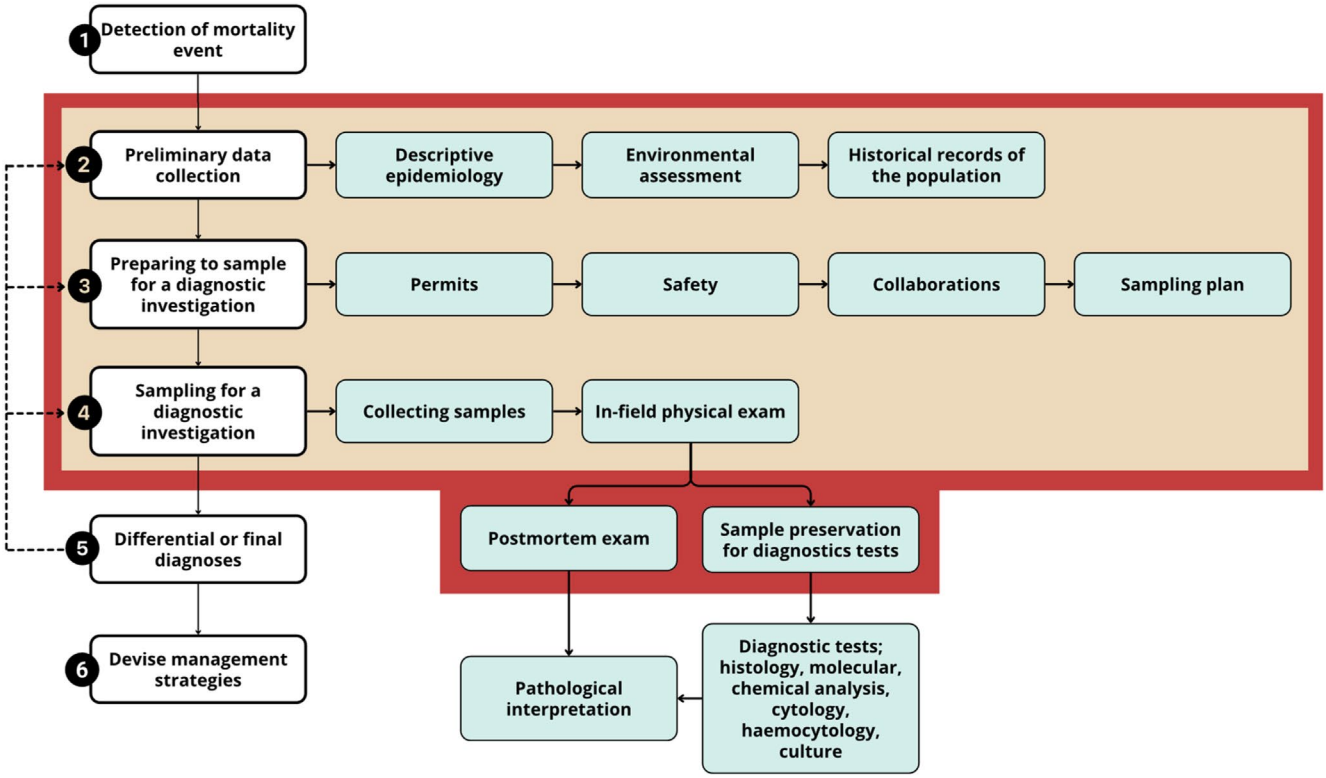

FIGURE 1 Comprehensive overview of a mass mortality event (MME) diagnostic investigation. Numbered, white boxes show general steps, while green boxes show the sub-steps. The dotted arrows represent the cyclic nature of an MME investigation, where results from one sampling inform future sampling and analysis. In the simplest iteration of an investigation, field biologists can collect whole, live mussel samples and rapidly transfer them to a laboratory equipped for receipt and comprehensive processing. In this case, the field biologist must complete the brown area of the flow chart before sending the mussel samples to the laboratory for processing. The red area shows the parts of the procedure that, if a specialised laboratory is not available to do comprehensively, may have to be conducted by the field biologist. This paper covers the core steps of these procedures, equipment and methods necessary to prepare field biologists for conducting sampling under either the brown or red area scenarios.

2 | PRELIMINARY DATA COLLECTION

Although the focus of this paper is sampling for a diagnostic investigation, a basic set of preliminary data (Table 1) derived from field investigations is necessary to ensure the appropriate samples are collected, help prioritise diagnostic tests, and enable the interpretation of laboratory findings. The preliminary data could be collated from previous surveys or collected during or before sampling. At a minimum, these preliminary data should include the species and age class affected/unaffected, an approximate percentage of mortality and number of mortalities observed, a timeframe of mortality, any available information about the environmental circumstances (e.g. extreme flow, temperature and pH) that were observed in the water body before the MME, wider environmental changes noticed at the site (e.g. construction activities) and the presence or absence of mortalities in other taxa. However, first responders should not be dissuaded from collecting all the relevant data they can, even if it is not everything on the above list.

To quantify the extent of mortality, timed searches at each site can be undertaken, recording the number of healthy mussels, moribund mussels, dead mussels, and fresh shells found. Fresh shells typically have a clean and shiny nacre (refer to Southwick and Loftus (2017) for further information on assessing shell condition). For species that burrow, it may be necessary to use quadrats and carefully excavate the sediment in the quadrats (e.g. as in Brian et al., 2022; Smith et al., 2001) because moribund mussels may be less able to burrow and may disproportionately occur on top of the sediment. The mortality may not be evenly distributed, so searches should be conducted at multiple sites. However, care must always be taken not to spread any biological or chemical agents by proceeding from upstream to downstream (Section 3.4). For more information on quantifying aquatic MMEs, refer to the work of Marchowski et al. (2024) and Southwick and Loftus (2017).

Age can be estimated by measuring shell size, counting shell annuli (where they occur), and then using relevant published von Bertalanffy growth curves (e.g. as in Ollard & Aldridge, 2023).

In addition, it is beneficial to collect data regarding the mollusc community characteristics (e.g. age structure, population density, species diversity, and spatial extent of the mussel bed) (for detailed examples refer to Cummings et al., 2016), hydrological conditions of the water body (flow rate or discharge, water level), land use practices (for example, categorisations refer to p. 14 of British Environmental Agency, 2003), noticeable erosion (e.g. landslips) around the site, locations and sources of upstream discharges (e.g.

TABLE 1 Essential preliminary data to collect or collate during a diagnostic investigation of a freshwater mussel mass mortality event (MME).

Parameter	Purpose	Methodological guidance
Prevalence and timeframe of mortality	Knowing the proportion of mussels affected by the MME provides key information about its extent and likely effect on the population Knowing the timeframe of mortality places the event relative to surrounding environmental changes and gives insights about the causative agent regarding its speed of action	Section 4.3 (for diagnosing moribund vs. healthy mussels), Marchowski et al. (2024) and Southwick and Loftus (2017) (for quantifying dead mussels)
Size/age	Knowing whether the MME disproportionately affects old or young mussels could help diagnostics and predict long-term population outcomes	Ollard and Aldridge (2023) (for estimating age from size)
Mortality in other taxa	Knowing the specificity of the MME helps diagnostics and predict community-level effects	At least note any mortality in other taxa, refer to Marchowski et al. (2024) for quantifying mortality in other aquatic taxa (e.g. fish, snails)
Impacted mussel density and abundance	Helps provide information about the total impact of the MME and insights into possible causes, such as the potential for transmission of infectious agents	Refer to examples in Smith et al. (2001) and Brian et al. (2022)
Obvious environmental changes	Provides information on potential causes	Record any obvious environmental changes such as habitat destruction by humans (e.g. construction activities)

wastewater treatment plants, industrial plants, feedlots), signs of pollution around the site, and any information about previous MMEs. Ideally, if invasive bivalves (e.g. *Corbicula* spp. or *Dreissena* spp.) have been recorded at the site, this should be noted and their abundance roughly quantified. It should be noted that in some cases it may be necessary to obtain a permit for the collection of these preliminary data.

3 | PREPARING TO SAMPLE A MASS MORTALITY EVENT FOR A DIAGNOSTIC INVESTIGATION

Mass mortality events must be sampled rapidly after their detection to ensure that adequate samples of moribund individuals can be found. To facilitate rapid sampling, it is important to be prepared with appropriate permits, safety procedures, collaborations and a sampling plan.

3.1 | Permits and legal cases

The sampling team should contact the appropriate state, federal, provincial, or equivalent natural resource agency to acquire any permits necessary for the handling, sampling, and collection of mussels from the site. Ideally, generalised sampling permits could be prepared in advance in case an MME were to occur because rapid sampling is essential. Considering that in many countries sampling water and sediment does not require a permit, in cases where permits for mussel sampling are not immediately forthcoming, water and sediment could be sampled immediately using the procedures we outline. Local research teams should be aware of what sampling can be done without a permit and this knowledge used to maximise the amount of sampling that can take place immediately after detection of a possible MME. In the event of a legal investigation, it is imperative to understand and follow procedures for the proper documentation of the collection, transport, storage, and analysis of legal case specimens (e.g. evidence tags or chain of custody forms) (Southwick & Loftus, 2017). Where the MME has happened across many taxa (e.g. fish), it may be necessary to coordinate with other research teams.

3.2 | Safety and training

Safety concerns are similar to those of other field investigations and include environment, equipment use, hazardous materials, health, work hours, and dealing with the public. The sampling team should follow their agency's standard operating procedures for safety and be trained in the proper methods for the collection, handling, species identification, and transport of freshwater mussels.

3.3 | Collaborations

A comprehensive MME investigation typically involves collaborations with other laboratories to aid in sample analysis, conducting postmortem examinations and sample preservation steps, and/or field collection of more specialised additional samples. Such collaborators can be sought by contacting local government animal health laboratories, fish health laboratories, wildlife disease surveillance programs, veterinary diagnostic laboratories, local universities, or relevant region-specific professional organisations.

3.4 | Sampling plan

A thorough sampling plan includes determining the sampling design, sampling sites, sampling direction, samples to collect, order of sampling, timeframe for sampling and sample processing, decontamination procedures, and gear needed.

As part of the sampling plan, establish an appropriate sampling design. A before–after control-impact (BACI) design is optimal (Christie et al., 2019) but it is likely not feasible because it would require prior in-depth monitoring and sampling of populations before an MME occurs. Rather, to determine which pathological findings or causal agents are unique to an MME, moribund individuals along with apparently healthy control individuals at the impacted site, as well as healthy control individuals from an unimpacted control site, should be sampled (Figure 2). Moribund individuals rather than dead individuals should be prioritised to limit confounding factors due to decomposition (Knowles et al., 2023; Waller & Cope, 2019). The healthy control population should be as similar to the impacted population as possible, for example, from an unimpacted, isolated, upstream location or tributary. Alternatively, mussels could be sampled from a similar nearby waterbody. If no suitable control sites are available within the watershed, a different watershed or a temporal control (e.g. sampling after mortality ends as well) could be considered. The sampling plan should also include a comparison of external environmental samples and parameters, including water samples, sediment samples, and temperature between the impacted site and unimpacted site (Figure 2). Sampling regimens similar to these recommendations have been applied to studying freshwater mussel MMEs previously and reflect a typical epidemiological case–control study design (Richard et al., 2020, 2021). If possible, repeat samplings over time of both the control and the impacted site should be undertaken, including sampling after an MME has ended (assuming mussels remain) (Leis, Dziki, Richard, et al., 2023; Richard et al., 2022).

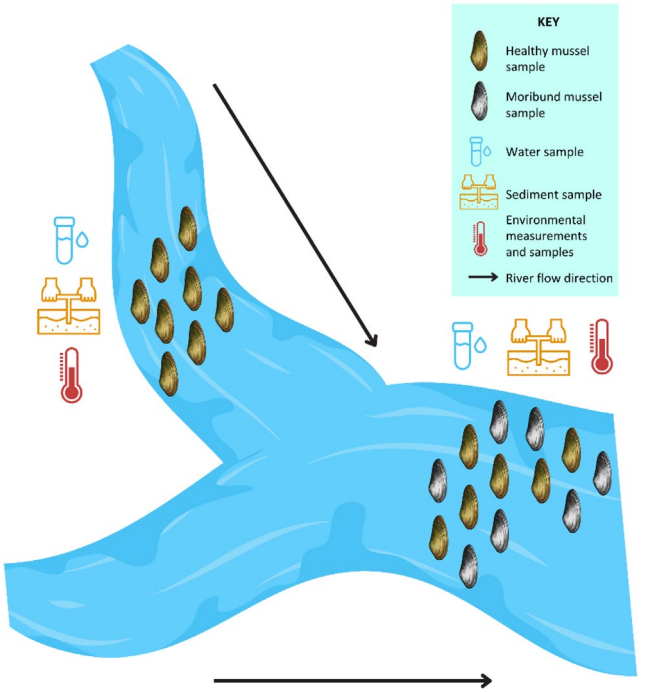

FIGURE 2 Outline of an example freshwater mussel mass mortality event sampling design. Mussel samples (Section 4.2), both healthy and moribund (see Section 4.3 and Figure S1 for distinguishing healthy from moribund individuals), water samples, sediment samples and additional environmental measurements (Section 4.1) should be collected from the site of the MME. In addition, samples from an unimpacted population not experiencing mass mortality should be collected. Such sites could be an upstream location of the same river, an upstream tributary (as depicted) or a nearby waterbody. Environmental measurements and samples could also be taken both upstream and downstream of the sampling sites.

Sampling should be undertaken in a downstream direction (if in a river) and at unimpacted sites without known MMEs before impacted sites to avoid the spread of biological or chemical agents. If an unimpacted site must be sampled after an impacted site, a separate set of gear for impacted and unimpacted sites should be used. If this is not possible, gear should be decontaminated before entering the unimpacted site with a 1% Virkon solution or a 10% bleach solution, according to the manufacturer's instructions. Similarly, gear should be decontaminated between days and discrete sampling sites.

Rapid response to an MME can also be facilitated by the preparation of a response kit that contains the necessary sampling supplies (File S1) and can be stored on hand for sampling at short notice. Preparation of the required equipment is especially important if sample processing (Section 4.4) must be undertaken in the field because in this case it will be necessary to bring the required preservatives sampling (Section 4.4, File S1).

4 | SAMPLING A MASS MORTALITY EVENT FOR A DIAGNOSTIC INVESTIGATION

Considering the complex interactions between multiple stressors (any biological or physical factor that negatively affects the physiology of an organism; for example, etiological agents such as parasites or environmental factors such as high temperature) involved with MMEs, a standardised protocol that involves a cross-disciplinary approach integrating novel and traditional techniques to investigate a range of potential causes is essential to provide a deeper understanding of MMEs (Carella et al., 2023). To facilitate this, we outline a procedure for the collection and preservation of samples from a freshwater mussel MME. Following our procedure, the collected samples can be used for a variety of analysis methods (Table 2). If resources are limited, priority should be placed on collecting and processing mussel samples (Sections 4.2–4.4).

4.1 | Collecting environmental parameters and samples

As time permits, environmental parameters and samples can be recorded and collected. There are many analytical options for

TABLE 2 Outline of the diagnostic tests used when studying freshwater mussel mass mortality events (MMEs).

Diagnostic test	Information obtained	Advantages	Disadvantages	Target organ/s to sample[c]	Long term preservation of samples	Are there non-lethal sampling options
Essential, sample preparation is part of the core procedure						
Molecular (e.g. metagenomics or qPCR)	Identification of both micro and macro parasites	Holistic view of microbes, Can be non-destructive	Expense, general lack of knowledge on microbes/viruses that are pathogenic to mussels, difficult to interpret the significance of a disease without pathological assessment, errors in species identification.	Haemolymph[a], Biopsies from multiple organs	Frozen at −80°C (or in liquid nitrogen[d])	Yes
Chemical analysis (e.g. mass spectroscopy, colourimetric assays)	Identification of substances toxic to mussels such as metals, pesticides, PAHs, ammonia, and biotoxins (e.g. microcystin)	Very reliable quantitative result, Several toxic substances (e.g. metals, pesticides) have well-established analyses	Needs a relatively large amount of tissue (0.5–1 g), destructive, likely requires pooling tissues, a large range of different analyses for different chemicals necessitates some prior hypothesis regarding what to target, which will likely involve consultation with an expert laboratory, clear evidence of the involvement of pollutants in mussel declines is difficult to establish (e.g. Woolnough et al., 2020), Health impacts of many toxins (especially biotoxins) are poorly understood, most lack established thresholds for healthy levels in mussels, and synergistic adverse effects have barely been considered.	Tissue homogenates of parts of or whole mussels	Frozen at −20°C or colder	No
Histology	Visualisation of etiologic agents (macro and most micro parasites) and lesions associated with pathology, Diagnosis of specific diseases	Only method that can specify type of disease according to pathological process, thus, helpful in determining the significance of a broad range of potential causes, Samples preserved for histology can be utilised for other methods such as TEM if prepared correctly	Destructive, requires toxic chemicals for preservation, requires specialised processing and expertise to interpret histological sections.	Sections of the key organs of the body.	In 10% neutral buffered formalin or similar[b]	No
Non-essential, ancillary methods that can be done if specialists are available						
Haemocytology	Identification of disseminated neoplasia, abnormal haemocyte morphology or composition, and pathogens in the haemolymph	Non-destructive, gives results relatively quickly	Requires specialist knowledge for sample collection and interpretation, requires immediate processing, difficult to obtain equipment/reagents in time, references ranges are not well-established, characteristic haemocytic responses to different types of host injury are not well established.	Haemolymph	Immediately process onto slides	Yes

TABLE 2 (Continued)

Diagnostic test	Information obtained	Advantages	Disadvantages	Target organ/s to sample[c]	Long term preservation of samples	Are there non-lethal sampling options
Culture	Identification of microbial pathogens	Provides isolates of microbes which can allow future experimental infection and/or the sequencing of longer fragments with Sanger sequencing, can be non-destructive	Understudied aquatic pathogens may require unusual culture conditions, requires specialist knowledge for sample collection to prepare plates for immediate sample processing, large biases in what will grow on the plates mean this method should not be used in isolation.	Haemolymph	Immediately add to culture plates	Yes
Cytology (e.g. wet mount preparations of tissue clippings or scrapings and/or stained preparations of tissue imprints)	Identification of surface-associated protistan parasites, macroparasites (e.g. trematodes), fungi, and large bacteria, Identification of gross abnormalities of tissue, cellular infiltrates, and disseminated neoplasia	Gives a rapid indication of potential problems, inexpensive, conventional method to detect organisms that may dissociate from surface tissues when in fixative, stained preparations can be stored for later analysis	Wet mount preparations require specialist knowledge for sample collection and interpretation requires immediate processing and identification of parasites/abnormalities, most ectoparasites are not expected to have a large causative role in MMEs, stained preparations require an extra step to prepare slides during sampling and some level of specialised knowledge.	Multiple organs and sample types (scrapings, fluid, tissue clips, etc)	Immediately process	Yes
Non-essential, alternative use for haemolymph or tissue collected if enough remains after other diagnostic methods						
Biomarker assays (including clinical chemistry)	Gives insights into general health status of the mussel through various potential assays such as, metabolomics, protein content, glucose content, or acetylcholinesterase activity	Screens for biomarkers of health and organ function, provides a finer characterisation of health status than visual symptoms	Limited reference ranges are available for freshwater mussels, Does not provide specific diagnostics as multiple stressors can impact the same parameter.	Haemolymph or tissue biopsies	Frozen at −80°C	Yes

Abbreviation: TEM, transmission electron microscopy.

[a]Haemolymph can be drawn non-lethally in the field if non-lethal methods are required. However, lethal sampling is recommended for other diagnostic procedures. Refer to the text (Section 4.4) for more detail on haemolymph sample transport and preservation if non-lethal sampling is required.

[b]10% neutral buffered formalin is the most widely used solution for routine fixation. Other fixatives can be used (Section 4.4.1). It is recommended to wash samples out of formalin into ethanol after at least 24–48 h of fixation (Section 4.4.1).

[c]A description of the best way to combine samples from these organs for the diagnostic tests can be found in the main text (Section 4.4).

[d]Other options are discussed in Section 4.4.

environmental sampling that may require unique storage and handling, and a comprehensive review is beyond the scope of this paper. We provide some suggestions for parameters to measure but highlight that local context and contact with relevant specialists will determine the best environmental parameters or samples to take at a given MME.

Basic environmental factors (e.g. temperature, salinity or conductivity, dissolved oxygen (DO), ammonia and pH) can be measured with appropriate probes and meters on-site, and upstream and downstream of all sampling areas. Many environmental factors are subject to significant diurnal fluctuations in the aquatic environment (Bridgewater et al., 2017) and should be measured multiple times throughout the day if possible. At a minimum, measurements should be collected contemporaneously with mussel samples.

Water samples (~1 L) can be collected, placed on ice and transferred to a laboratory for the measurement of alkalinity, hardness, turbidity, nutrient concentrations, suspended solids, or other parameters if desired (Bridgewater et al., 2017). These water samples should ideally be collected at representative points in the sampling reach (e.g. up and downstream, near any effluent sites or springs, etc.). If water quality issues are suspected as a likely cause, consult a specialist for additional sample collection considerations.

Potentially harmful chemicals (e.g. metals or biotoxins) from the environment can also be sampled by collecting three replicates of approximately 2 L of surficial sediments and water. Multiple representative locations should be sampled. Unless the event involves chronic toxic contamination or sample collection occurs contemporaneously with the onset of the event, harmful chemicals may be difficult to detect in water samples (Southwick & Loftus, 2017). In contrast, sediment samples can contain residual contaminants after the event has passed (Southwick & Loftus, 2017). For many chemicals, amber glass jars are ideal for collection followed by freezing at −20°C if storing long term (ensure to leave space for water expansion during freezing). However, if a chemical is a suspected cause of the MME, the relevant departments of health or environment should be informed and a specialised laboratory should be consulted. The details of environmental chemical sample collection may depend on the target chemical and testing strategy used by the specialised laboratory (Campisano et al., 2017). For example, it is advisable to avoid storage containers that may leach contaminating compounds into the sample.

If available, a flow meter can be used to measure the flow rate. Otherwise, flow rate and water-level data may be available from in-stream monitoring gauges. During analysis, the environmental data gathered during the MME can be compared with any historical data collated from records (Section 2) to detect any anomalies that may have contributed to mortality.

Algae and cyanobacteria can produce harmful toxins or otherwise fatal hypoxic conditions during periods of post-bloom decomposition. Changes in water colour, unusual odours, and the presence of non-filamentous surface scum should be noted because this will be useful for attributing an MME to algal and cyanobacterial blooms (Hudnell, 2008).

Environmental samples for molecular analysis (e.g. PCR or metagenomics) can be collected. Water (three replicates of approximately 1 L) can be collected and filtered using 0.22 µm Sterivex™ (or similar) filters (Merck, Germany). Ideally, both filters (containing cellular microbes) and filtered water (containing viruses) should be frozen at −80°C within 24 h of collection and kept on ice until freezing. If 1 L cannot be filtered through a 0.22 µm filter due to suspended particles, a prefilter through an approximately 20 µm filter can be used. Water samples can be obtained directly from the waterbody with syringes before filtering or first collected in a bottle before filtering. If the bottles for collecting and storing water are not sterile, they can be sterilised with bleach for 5 min before use. The bleach can be rinsed out with sterile water or water from the specific site of the sample collection. Three replicates of 1–5 mL of sediment can also be collected in a 5 mL microcentrifuge or conical tube and frozen the same way as the water samples. Gloves should be worn when collecting the water and sediment samples and the samples should be collected upstream of your body in flowing water.

4.2 | Mussel collection

The following sections discuss sample collection and processing using lethal methods. Lethal sampling of mussels allows for an analysis of the whole animal, which is necessary for the detection of some stressors (e.g. pollutants) and histological analysis, a fundamental aspect of all MME investigations. More stressors can be analysed from one individual when taking lethal samples, simplifying the sampling procedure, reducing the overall sample size and allowing the investigation of associations between different stressors. If lethal sampling is completely restricted due to the protected nature of the species, non-lethal sampling methods can be considered (refer to Section 4.4.2).

Mussels can be found using a bathyscope and collected from the water body by hand. However, if the water body is too deep, rakes, dredges, snorkelling or diving may be required to find and collect mussels. Ideally, individual notes about each mussel's condition and behaviour in the water body, on removal from the water body and while handling, would be recorded before the mussel is placed in a uniquely labelled bag (refer to File S2 for an example datasheet). However, in many situations, such detailed notetaking may not be possible. Instead, mussels can simply be separated into healthy and moribund individuals with notes on the criteria used for the designations recorded (Section 4.3). For transport to a suitable place for processing, individual mussels can be placed in separate bags in a cooler or container of ice with a layer of cardboard or towels between the mussels and the ice to ensure the tissue does not freeze. Photographs should be taken to document the site, the mortality of mussels in situ, and the appearance of each sick and healthy mussel sampled.

During a day of sampling of an MME, the exact number of samples collected will vary depending on factors such as permit restrictions, the size of the team, the timing of sample collection relative to

the MME (e.g. if mortality has mostly ceased, good moribund specimens may be hard to find among shells), other variables to collect (e.g. water samples or sediment samples), the method of sample processing (e.g. lethal removal of mussels to a lab for processing, lethal sampling of mussels in the field, or non-lethal processing in the field), the travel required to reach the site, water body conditions (depth, water clarity), mussel density and the weather conditions on the day. A similar number of moribund individuals, apparently healthy individuals from the impacted site, and apparently healthy individuals from the control site should be collected (Hewson et al., 2024). Depending on the above factors, approximately 10–20 individuals in each group can be an achievable goal.

Diagnostic value rapidly decreases as the time since death increases due to decomposition; therefore, sampling dead mussels is often avoided (Knowles et al., 2023; Waller & Cope, 2019). Despite this, dead mussels can be sampled in addition to moribund mussels, especially when moribund mussels are in short supply. However, dead individuals should not be sampled instead of available moribund mussels. Moribund and healthy controls should be collected from all impacted species.

4.3 | In-field physical exam

Physical examination is a standard component of any diagnostic investigation and is undertaken to classify morbidity and better characterise the nature and duration of disease and involved organ systems. However, like many invertebrates, physical exam methods and metrics in mussels are presently limited. The classification of health is generally limited to moribund versus not moribund, and studies compare these groups to identify significant associations from coincidental (Alfjorden et al., 2024; Da Silva Neto et al., 2024; Leis et al., 2019; Leis, Dziki, Standish, et al., 2023; Richard et al., 2020, 2021; Wengström et al., 2019). Therefore, a field biologist will need to use a physical examination of a mussel to classify it as either moribund or healthy. Distinguishing between healthy, moribund and even dead mussels can be challenging; thus, the following provides guidelines for categorising the health status of mussels.

The moribund state of mussels progresses incrementally, resulting in a gradient of lost organ functions as tissues die. The shell adductor muscle can act independently and may remain responsive after other vital processes have stopped. Therefore, we recommend using a suite of indicators to determine health status. Healthy control animals will often be buried firmly in the sediment, rapidly respond to tactile stimuli, close their valves firmly when disturbed, and resist opening when challenged (Richard et al., 2020). In some cases, a healthy mussel may be near the surface of the sediment and unburied if moving. Gravid females of some species will be above the surface of the sediment to display and/or release glochidia for fish infection. They can be distinguished from moribund mussels by their active valve closure response to probing. Moribund individuals will often be sitting on the surface of the sediment showing abnormal behaviour. For example, they may display gaping valves, tightly closed valves without filtering activity, secretion of excessive mucus, and slow or no response to tactile stimuli (Curley et al., 2021; Knowles et al., 2023; Richard et al., 2020; Southwick & Loftus, 2017). Moribund mussels may be only able to close their valves slowly and weakly, or the valves may be easily opened and held open by hand when challenged. Typically, fully open valves indicate a dead individual, which can be collected, but these samples are suboptimal compared to moribund specimens (Section 4.2). It is not necessary to observe all symptoms in a mussel to classify it as moribund; rather, only a selection of symptoms will likely be observed in an individual moribund mussel. For reference images of mussels from MMEs, refer to Figure S1.

4.4 | Postmortem examination and sample preservation

In an ideal scenario, mussel samples collected in the field can be shipped to a diagnostic lab with expertise in mussel mortality investigation. In this case, a complete postmortem analysis can be accomplished, which may involve ancillary diagnostic methods that will not be discussed in detail in this paper. Such methods may include haemocytology, cytology of tissue preparations and microbial culture. Although these methods have benefits (Table 2) and could be included in a comprehensive investigation, they will likely be impossible unless there is access to specialist knowledge (for more, refer to File S3). Ideally, such collaborations are in place before sampling mussels (Section 3.3). However, mortality events can outpace abilities to identify laboratories that will perform the tests required for a comprehensive diagnostic investigation. Even so, the field biologist can still confidently undertake a core procedure to conduct a partial postmortem examination and preserve essential samples (Figure 1, steps in exclusively red box), facilitating diagnostic tests and pathological interpretations once collaborations have been found (Figure 1, steps outside the red box). This core procedure is detailed below. During this procedure, institutional guidelines for the euthanasia of bivalves should be followed.

4.4.1 | Core procedure

Depending on logistics and resources, the core procedure may be conducted in the field or in a laboratory. The laboratory is typically the preferred setting because it allows for more time to collect mussels when sampling, is generally safer, is conducive to more detailed tissue examination and provides better conditions for collecting molecular samples. The field setting may be preferred in remote locations where samples cannot be quickly transferred to the laboratory. If in the field, we ensured to prepare all the necessary material to preserve samples such as dry ice and formalin (refer to text below and File S1) before the sampling day.

Process mussels within approximately 24 h of their collection to ensure as little change in the microbial communities as possible.

During dissection, it is important to follow aseptic technique to avoid cross-contamination. For each mussel dissection, wear fresh, clean, sterile gloves and sterilise all tools with ethanol and flame or bleach followed by molecular-grade water between specimens. Use fresh needles and syringes for each mussel when drawing haemolymph. Sterilise the workspace and cutting board between mussel dissections (e.g. with bleach) and/or use large, sterile single-use Petri dishes for dissections. Use single-use sterile weighing trays for each mussel. File S2 contains a datasheet that can be used for recording all the below-suggested notes.

First, take measurements of the shell length, width, and height and note any external shell abnormalities. Next, extract haemolymph by opening the mussel with sterile paediatric nasal speculums, mussel tongs, or other tools, inserting a 25–26 gauge needle attached to a 1–3 mL medical syringe into the anterior adductor muscle, and slowly drawing fluid (Fritts et al., 2015; Gustafson, Stoskopf, Bogan, et al., 2005; McCartney et al., 2009; Raley et al., 2006) (Figure 3a). Haemolymph samples for molecular analysis should be immediately placed on ice or at 4°C and stored at −80°C as soon as possible on the day of collection.

Subsequently, cut the adductor muscles with a sterile scalpel and open the mussel (Figure 3b). Note any soft tissue abnormalities, record the condition of the marsupial gills (i.e. gravidity) in females, and take a photograph of the valves open with tissues in situ to show the condition of the mantle, gills, and visceral mass. Then cut the soft body of the mussel out of the shell by cutting the remaining muscles that attach the visceral mass to each side of the shell (retractor and protractor muscles), running the scalpel under the mantle along the pallial line to free the mantle from the shell without ripping it, and carefully cutting the remaining muscles and ligaments attaching the mussel body to the shell from around the hinge area (Figure 3c). Record the soft tissue wet mass and shell mass and note any abnormalities on the

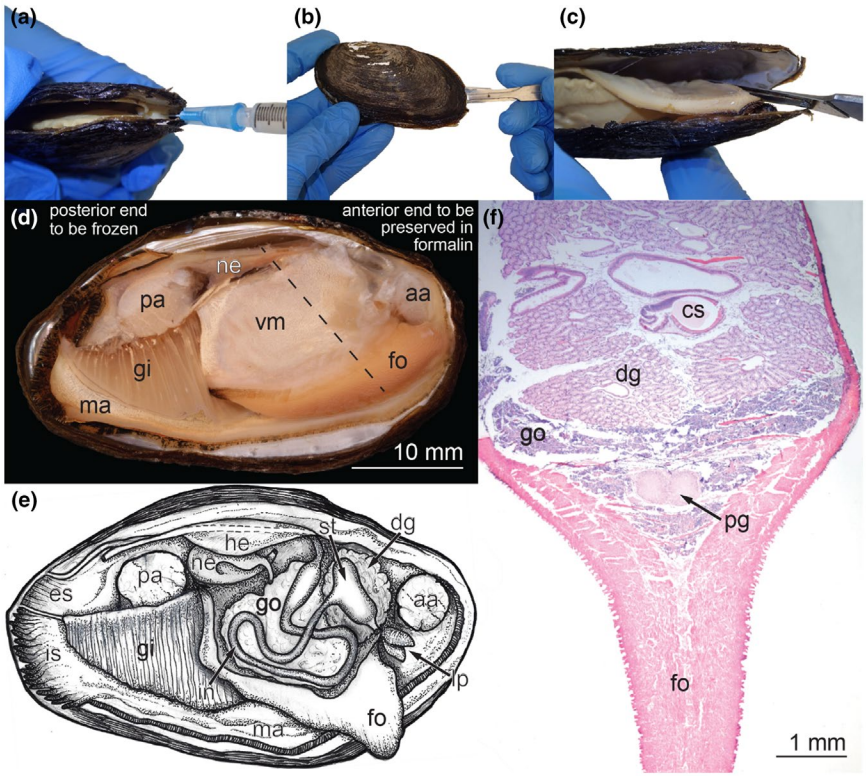

FIGURE 3 Processing mussel samples from a freshwater mussel mass mortality event. (a) A 25-gauge needle attached to a 1-mm syringe inserted into the anterior adductor for haemolymph extraction. (b) A scalpel inserted into the mantle cavity for the purpose of severing the posterior adductor. (c) A scalpel running along the pallial line to remove the mantle from the shell. Note that (a–c) shows a formalin fixed *Elliptio complanata* specimen for visualisation purposes. When following the core procedure for sample processing, the tissue will not be fixed at this point. (d) A *Cambarunio nebulosus* with the valve, mantle and gills removed from the right side showing the anterior adductor (aa), foot (fo), visceral mass (vm), nephridium (ne), posterior adductor (pa), gills (gi) and mantle (ma). The dotted line represents the approximate location where a transverse section may separate the visceral mass into two portions that each contain digestive gland and gonad for further study. The anterior portion should be placed in 10% neutral buffered formalin or an alternative fixative for histology. The posterior portion should be frozen at −80°C for metagenomics and/or chemical analysis methods. (e) An illustration of the major external and internal organs and tissues of a freshwater mussel including the anterior adductor (aa), labial palps (lp), foot (fo), mantle (ma), gills (gi), incurrent siphon (is), excurrent siphon (es), posterior adductor (pa), digestive gland (dg), stomach (st), intestine (in), gonad (go), nephridium (ne) and heart (he). Figure by Danielle Marichal, WaterFront Center. (f) A transverse histological section of *Cambarunio nebulosus* showing the foot (fo), pedal ganglion (pg), gonad (go), digestive gland (dg) and crystalline style (cs).

inside of the shell. Store the shells in a cool dry area because they can be used later for various applications (e.g. ageing, contaminant analysis, and deformity examinations, among others).

To prepare the tissue for preservation, place the soft body on a sterile surface and make a transverse section near the anterior end of the mussel at approximately the widest portion of the visceral mass where the gonad-digestive gland junction is located (Figure 3d shows the location of this cut). This cut should be made such that the anterior and posterior sections contain a part of most of the major organs (digestive gland, gonad, foot, gills, and mantle; Figure 3e). In healthy mussels, the digestive gland (at the anterior end of the visceral mass) will often have a greenish colour and the gonads (posterior to the digestive gland, in the mid-visceral mass) a pale brown colour, which can be used to ensure that parts of both tissues are on either side of the cut. However, in sick mussels distinguishing these tissues is more difficult because they will likely both be shades of brown. If in doubt, have tissue of each organ on the anterior side of the cut.

Place the posterior end of the mussel in a −80°C freezer. From these tissues, molecular analysis including PCR or metagenomics and/or chemical (e.g. heavy metal, organic pollutant, pharmaceutical, or biotoxin) analysis methods can be undertaken. This tissue can be stored in a sterile plastic container such as a 50 mL conical tube. However, as with environmental samples, if intending to undertake chemical analysis, a specialised laboratory should be consulted as particular storage containers, sampling methodologies, and preservation conditions may be required depending on the target chemical analyte.

Place the anterior end of the mussel into 10% neutral buffered formalin for histology (or an alternative histological fixative, refer to Howard and Smith (1983)) (Figure 3f). If gravid and the mussel anatomy is such that the marsupium is not included in the anterior sample, ideally, collect a 3–4 mm strip of marsupium and add it to the fixative as well. The fixative volume should be approximately 15–20 times greater than the tissue volume (Carson & Cappellano, 2019). A sufficient volume of fixative to allow the tissue to float freely is essential.

Some may find it easier to make the transverse section while the mussel is still in the shell before cutting all the attaching ligaments. This would allow the anterior end of the mussel to be preserved in its shell, which will maintain the tissue structure better. However, making a clean and precise transverse cut in the shell can be difficult due to the curvature of the shell and the tough ligaments.

The recommended fixation time varies depending on the fixative used (Howard & Smith, 1983). For formalin, 24–48 h is typically advised. If the visceral mass of the specimen is thicker than 1 cm, serial transverse sections or small incisions at 5–10 mm increments should be made to allow for sufficient penetration of the fixative. To avoid tissue shrinkage artefacts and to allow for subsequent molecular analysis on histological preparations (such as in situ hybridisation or PCR), after fixing, place tissues in a graded series of ethanol solutions up to 70% ethanol for approximately 12–24 h per change (e.g. 30%, 50%, 70% ethanol) (Carson & Cappellano, 2019). The tissues can remain in 70% ethanol indefinitely but should be regularly checked and topped up with ethanol in case evaporation has occurred.

Samples to be stored in the −80°C freezer (haemolymph and the posterior end of mussel body) should be placed in the freezer as soon as possible. After collection, samples can be initially stored on dry ice, in a −20°C freezer, or on ice (for a few hours at most), until they can be moved to the −80°C freezer for long-term storage. Liquid nitrogen is a suitable alternative for long-term storage. If the samples are processed in the field (not recommended unless it is impossible to get mussels to a lab within 24 h) or the tissues to be stored at −80°C require further overnight transport to a different location, then these tissues should be transported on dry ice or in liquid nitrogen.

4.4.2 | Non-lethal sampling

In some cases, extensive lethal sampling may not be possible. Thus, to sufficiently sample individuals from such MMEs, non-lethal methods can be used either solely or to complement lethal methods and increase the total number of samples. Do not use non-lethal methods as an alternative to following the core procedure if it is possible to take lethal samples because non-lethal samples have limited diagnostic value compared to full tissues preserved according to the core procedure (Section 4.4.1). If undertaking non-lethal sampling, additional time will be required on the day of sampling to process samples in the field.

When sampling larger mussels (>5.5 cm), small foot or mantle biopsies of 3 mm^2 to 1 cm^2 (depending on the mussel size), haemolymph (0.5–1 mL) or gonadal fluid (<200 µL, reduce the volume in small individuals) can be taken non-lethally (Berg et al., 1995; Fritts et al., 2015; Gustafson, Stoskopf, Bogan, et al., 2005; Karlsson et al., 2013; McCartney et al., 2009; Naimo et al., 1998; Raley et al., 2006; Saha & Layzer, 2008; Tsakiris et al., 2016). The only combination of non-lethal biopsies shown to be non-lethal when used on the same individual is haemolymph withdrawal with foot biopsies (Fritts et al., 2015).

When collecting haemolymph non-lethally, follow the procedure outlined above (Section 4.4.1), taking additional care not to open the mussel too far and insert the needle only once. To take tissue biopsies, specific biopsy tools (such as 3 mm oval-cupped jaw biopsy forceps (Fritts et al., 2015) or biopsy punches) or a pair of small sharp scissors can be used. When collecting gonadal fluid, insert an 18–21-gauge needle preloaded with approximately 50 µL of sterile water into the gonad through the foot. After injecting the water, gonadal fluid can be withdrawn.

Preserve non-lethally collected samples in the field for molecular analysis immediately by adding them to dry ice. Alternatively, non-lethally collected samples can be taken on the day of collection on ice to a −80°C freezer. If more days are required until the samples can be frozen at −80°C, record the time spent on ice before freezing. If it is not possible to access dry ice or a −80°C freezer within a day of sampling, then RNAlater® (Thermo Fisher Scientific, Waltham, U.S) (or similar preservative) can preserve solid biopsies (foot/mantle) samples for a week at room temperature (but preferably at 4°C)

before they are frozen on return to the lab. If using RNAlater with tissue biopsies, samples can be stored for 1 month at 4°C or indefinitely at −20°C. Gonadal fluid can be preserved for cytological methods by adding it to 300 μL of 10% neutral buffered formalin (Brian & Aldridge, 2020).

If the number of mussels or time is limited, the non-lethal sample type and preservation method to prioritise will depend on the nature of the MME and any pre-existing hypotheses about the cause (e.g. if a suspected parasite is localised to a certain organ).

It is inadvisable to attempt non-lethal sampling on small mussels (<5.5 cm) because increased mortality has been observed following haemolymph and/or foot biopsy sampling in smaller mussels (Fritts et al., 2015).

4.4.3 | If no moribund specimens can be found

It can often be difficult to sample mussels as an MME occurs. It may be that upon arrival at the site only apparently healthy mussels, shells, and a few fully dead specimens are found. In this case, dead specimens should be collected. Apparently healthy individuals should also be sampled either lethally or non-lethally (refer to Sections 4.4.1–4.4.2). Similarly, a control site with no MME should be sampled. Although samples from dead mussels are suboptimal and likely have lower diagnostic value, they can provide valuable information in cases where ideal samples are lacking.

4.4.4 | Alternative sampling procedures

The core procedure above (Section 4.4.1) is designed to optimise sample collection for a diverse range of stressors when limited moribund samples are available, which can often be the case. However, alternative sampling procedures might be considered if focussing on fewer stressors or if more than 20 moribund samples can be found. For example, initial depuration of samples before fixing may improve the quality of histological preparations. If the necessary bio-secure facilities are available and chemical analysis is not a priority, it might be preferable to collect haemolymph samples and small tissue biopsies before depurating mussels, collecting additional tissues for other desired diagnostic tests, fixing mussels with shells on in 10% neutral buffered formalin, and transferring mussels into ethanol after 48 h of fixation. In contrast, if focussing on chemical analysis, after consulting with specialists, it may be desirable to preserve some whole mussels immediately at −80°C because certain chemical analytes may not be adequately represented in only the posterior section of the mussel. If plenty of moribund specimens can be found, samples could be split between chemical analysis and pathogen analysis methods to allow for the depuration and freezing of separate whole mussels. It is outside the scope of this paper to discuss all the intricacies of every available option. Such discussions can be undertaken with a specialist, and our methods can be adapted to their recommendations.

Ultimately, the division of samples from which individuals and for which tests will depend on any pre-existing hypotheses of potential causes, the permits obtained for sampling (lethal or non-lethal), the number of mussels found at the site, the resources available, and any pre-existing specialist collaborations. Even so, conducting the above-explained core procedure (Section 4.4.1; summarised in Figure 4 and Table 2) will adequately preserve samples for specialised labs to conduct a wide range of diagnostic analyses (a brief primer on analysis methods can be found in File S3).

5 | DISCUSSION

Many MMEs are the result of multiple interacting factors (Carella et al., 2023; Soon & Ransangan, 2019); thus, when investigating MMEs, cross-disciplinary methods that simultaneously target a range of potential causes are needed. This is especially the case when suspicions of potential causes are weak, necessitating a broad search of many potential stressors. This paper has set out a systematic method to collect samples for the investigation of multiple potential stressors that could be causing an MME. Various case studies of MME investigations demonstrate the application and importance of procedures such as ours for sampling MMEs in freshwater mussels (Box 1) and other taxa (Box 2). Our procedures will allow researchers and managers who are not specialists in MME investigations to be prepared to sample an MME promptly and sufficiently so that adequate material can be preserved to facilitate diagnoses. Preparedness is essential when investigating MMEs because any delay in collecting and preserving samples could introduce artefacts such as opportunistic bacteria growing on dead tissue, which may confuse cause with effect.

Understanding the causes of MMEs is difficult. It may be that initial investigations using our methods fail to pinpoint a single cause. Often the diagnostic investigation will require multiple iterations following adjustments or additional tests based on previous results identifying additional stressors to investigate before a refined diagnosis is achieved. Sampling may occur too late to collect the ideal moribund specimens. Even if samples are correctly prepared and preserved, it could be that the causative agent has passed through the system and is no longer detectable in the environment or mussel tissues.

If sampling occurs soon enough to capture moribund mussels while the stressor is still present, our procedure may reveal common potential causes across multiple MMEs. This is an important step for understanding freshwater mussel MMEs further (Waller & Cope, 2019). Even so, our methods alone cannot provide definitive evidence of the cause of an MME. Rather, the methods can inform future investigations of the MME, such as experimental infections, to prove the causative role of an identified stressor in inducing mortality. These investigations could lead to the development and application of rapid specific tests for important pathogens (Leis, Dziki, Standish, et al., 2023), the avoidance of translocations of diseased or parasitised individuals (Brian et al., 2021), or the reduction of the impacts of multiple stressors (Tyack et al., 2022).

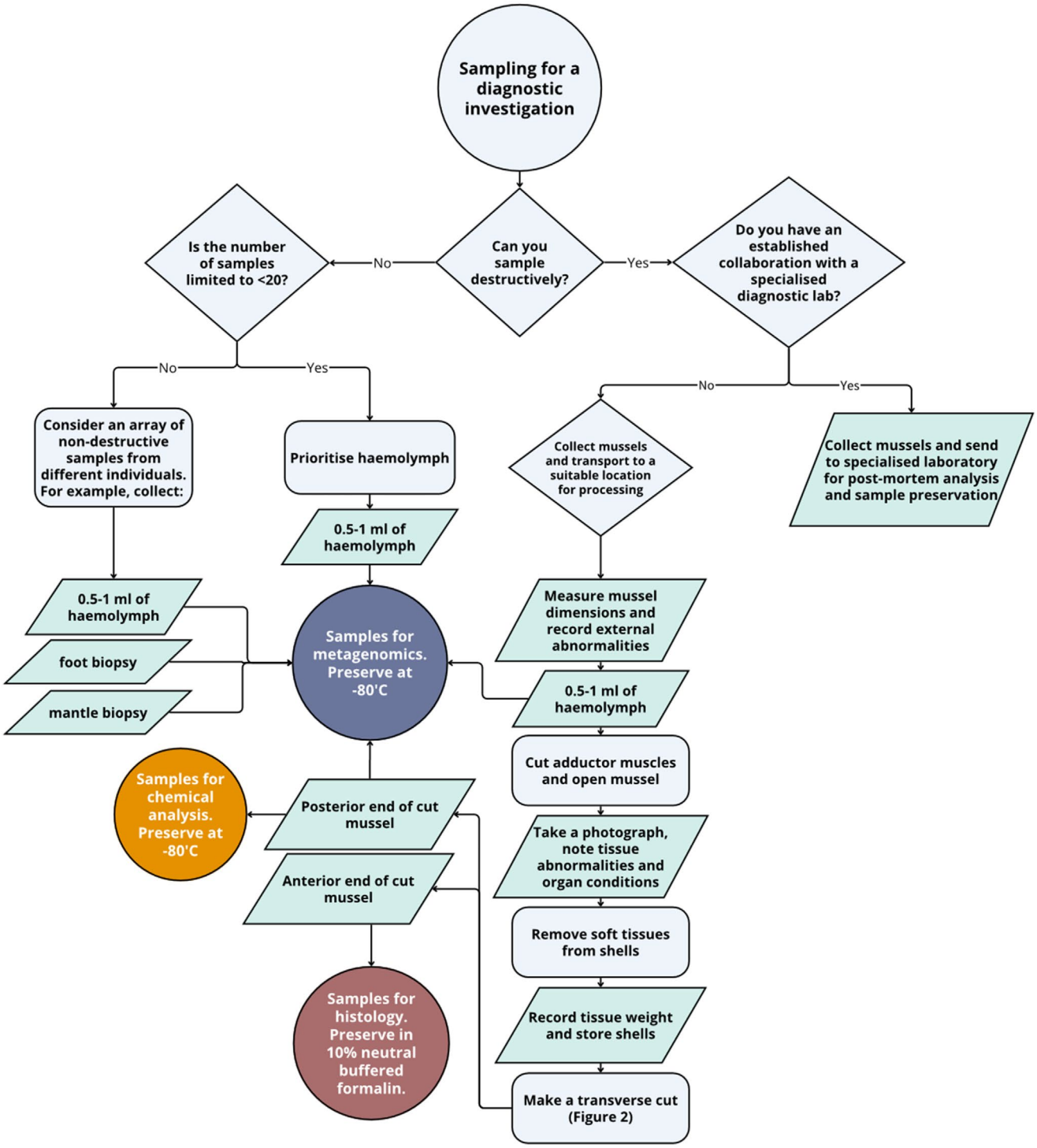

FIGURE 4 Decision tree and steps for the core sampling procedure for a freshwater mussel mass mortality event. For further details on additional sampling procedures available with specialist knowledge, what to do if no moribund specimens can be found, considerations for conducting procedures in the field, sampling groups, numbers and methodologies, see the main text. Grey diamonds show decisions, grey rectangles show steps to take, light green parallelograms display material or data collected from the mussel, multicoloured circles display the diagnostic test that the collected material can be used for and a brief note on sample preservation. For long-term storage of samples for histology (brown circle), washing into ethanol is recommended, see Section 4.4.1 for more.

Investigating MMEs should only be one part of the regular monitoring of populations. Indeed, a critical need for freshwater mussel conservation lies in the periodic application of the methods discussed in this paper to populations not experiencing MMEs or declines, especially those of high conservation status. This would help identify 'background' conditions and microbiota (Brian & Aldridge, 2023), better understand threats to mussel health, improve early detection of populations under stress, facilitate the establishment of proactive

BOX 1 Mass mortality event investigation case study one

Here, we provide an example of an ongoing freshwater mussel MME investigation that utilises methods akin to those we have outlined. In the Clinch River, USA, annual MMEs of freshwater mussels have been observed since 2016 (Da Silva Neto et al., 2024; Richard, 2018). Using the study design outlined above, research into these events has involved molecular methods using DNA extracted from frozen haemolymph (Richard et al., 2020, 2021) and bacterial isolates grown from the culture of species present in the haemolymph (Leis et al., 2019; Leis, Dziki, Richard, et al., 2023; Leis, Dziki, Standish, et al., 2023). This work revealed a consistent association between the bacterium *Yokenella regensburgei* and moribund individuals during active mortality events. Further histological research confirmed these findings and revealed lesions associated with *Yokenella regensburgei* infection of sufficient severity to explain the mortality of individuals (Da Silva Neto et al., 2024). However, the cause of these infections and the ultimate cause of the MMEs require further investigation.

BOX 2 Mass mortality event investigation case study two

Here, we place our standardised MME sampling procedure in the context of sea star wasting disease (SSWD), which began in the boreal summer of 2013 and led to massive declines of sea star (Asteroidea; Echinodermata) populations across the Pacific coastline, from Baja California to Alaska (Hamilton et al., 2021; Hewson et al., 2024). There was a widespread response to SSWD that involved both citizen scientists and biologists (Hewson et al., 2024), generating a large sample size. However, many of these first responders were ill-equipped to sample for an MME investigation, lacking appropriate protocols. Drawing from work describing lessons learnt during the investigation of SSWD thus far (Hewson et al., 2024), we highlight some examples where a standardised, readily available protocol such as ours could have been useful.

Sampling plan (Section 3.4): During the initial sample collections from SSWD events, there was a disproportionately large number of diseased animals collected without sufficient healthy controls (Hewson et al., 2024). Moreover, the majority of healthy controls collected were from the same sites as diseased individuals, with few from unimpacted sites. This lack of sufficient controls complicated the distinction between baseline conditions and preclinical infections and contributed to spurious conclusions about potential causative agents (Hewson et al., 2024). This emphasises the importance of following sampling designs akin to those outlined in our procedures, particularly sampling non-impacted sites, to ensure adequate comparisons can be made.

Postmortem examination and sample preservation (Section 4.4): Many samples initially collected were not preserved or transported appropriately (e.g. stored and shipped at temperatures insufficient to preserve microbial communities) (Hewson et al., 2024), limiting opportunities for comprehensive diagnosis and highlighting the importance of following appropriate protocols for sample preservation. Initially, a densovirus was proposed as the causative agent of SSWD based on molecular evidence. However, histopathological analyses completed at a later date did not support this conclusion (Hewson et al., 2024). This emphasises the importance of collecting samples for multiple analytical techniques, especially histology, and withholding conclusions until all the evidence can be comprehensively analysed.

To date, no definitive causative agent for SSWD has been identified (Hewson et al., 2024). Current hypotheses include environmental factors such as high temperatures, organic matter enrichment, and surface oxygen depletion, though there is insufficient data to comprehensively support any hypothesis (Hewson et al., 2024). We argue that a planned, standardised procedure for sampling MMEs that may occur in the future will aid in efficient and effective diagnosis, enabling successful response and management.

measures before MMEs occur and improve pathological interpretations during MMEs (Burcham et al., 2023). In particular, during regular monitoring, the application of biomarker assays (e.g. clinical chemistry or metabolomics) to measure health status could be considered (Devin et al., 2023; Fritts et al., 2015; Gustafson, Stoskopf, Showers, et al., 2005; Legrand et al., 2023; Putnam et al., 2023).

Biomarkers were not specifically considered in our MME sampling procedures because, although useful during regular monitoring, these assays are unable to unequivocally identify the chemicals or other stressors potentially responsible for mortality. This is because most parameters measured can be modulated by different classes of pollutants and are also influenced by environmental factors (temperature, food availability, etc.) (Devin et al., 2023). Therefore, biomarker assays are not an essential diagnostic tool to identify potential causes of MMEs. However, they can still serve as a valuable supplementary tool during an MME investigation, providing insights into mussel health status if there is sufficient haemolymph or solid tissue available. Considering the time, laboratory processing,

and expertise required to undertake biomarker assays, the development of a standardised, accurate, widely applicable, rapid, and non-destructive field test of mussel health would aid in diagnosing mussels at earlier stages of sickness and monitoring baseline conditions in healthy populations.

Our sampling methods are not restricted to only MMEs but can be applied to cases of unusual mortality. Freshwater mussel populations can be impacted by unusual mortality events that may not be considered 'massive' but involve atypical conditions concerning the mortality. For example, the mortality of one species among a community of other unimpacted mussel species, mortality in threatened or endangered species, temporal or spatial changes in mortality, or unusual pathological findings [e.g. deformed shells (Pekkarinen, 1993; Strayer, 2008)]. Our methods can be readily applied to these cases as well as MMEs.

By assembling into one place the range of widely used tools and protocols that are available, and by further tailoring the protocols towards a specific taxon and to address a specific need, we have produced an important framework for enabling fieldworkers to contribute to our understanding of the drivers behind the numerous MMEs within freshwater mussel populations. Although our focus is on freshwater mussels, the principles we outline can be applied to many taxa, both in wild and cultured populations. Indeed, the protocols would require relatively little modification to be applicable to studying MMEs in marine bivalves (Carella et al., 2023; Lupo et al., 2021; Soon & Ransangan, 2019) and may be especially useful in the management of bivalve populations within aquacultural systems which can experience catastrophic MMEs (Guillotreau et al., 2017; Pernet et al., 2016; Robledo et al., 2014; Soon & Ransangan, 2019).

In addition to further diagnostic investigations of MMEs using the procedures we have outlined, MME reporting should be improved and accelerated for freshwater mussels and across all taxa. Alongside the publication of MME reports in scientific journals, which can involve long delays between the initial report and the data becoming publicly available, we encourage the development of an accessible database for reporting MMEs as soon as they are observed. The creation of such a database first requires a standardised, graded MME definition and reporting framework. Synthesising MME reports in an accessible database would improve MME response and monitoring, as well as inform wider population- or species-level threat assessments.

6 | CONCLUSIONS

With the ongoing rise in the frequency of MME reports, there is a pressing need for an increased capacity to promptly respond to and investigate such occurrences, particularly in understudied species that lack commercial significance or charismatic appeal (Hamilton et al., 2021). We have bolstered this capacity by delineating sample collection and preservation methods that can be used by biologists and conservationists observing MMEs of freshwater mussels, an important ecosystem engineer with a concerning and declining conservation status. Considering the wider application of our methods to other taxa, the protocol we have outlined has the potential to inform the investigation of MMEs in a range of species, enhancing conservation efforts across diverse ecosystems.

AUTHOR CONTRIBUTIONS

Daniel Alan Cossey led the writing of the manuscript. Daniel Alan Cossey, Michelle Marie Dennis, Jordan Richard, Camilla Della Torre, Andrew McElwain, Diane Lynn Waller, Susan Knowles, Joshua Ian Brian, Eric Leis, Erika Astrid Virginie Burioli and David Christopher Aldridge contributed to conceiving the ideas, structuring the information and providing material for the drafts. All authors gave final approval for publication.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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DATA AVAILABILITY STATEMENT

No data or code were used.

DISCLAIMERS

Any use of trade, firm or product names is for descriptive purposes only and does not imply endorsement by the US Government. The findings and conclusions in this article are those of the authors and the US Geological Survey and do not necessarily represent the views of the US Fish and Wildlife Service.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Figure S1: Examples of freshwater mussels from mass mortality events.

File S1: Freshwater mussel mass mortality event response kit.

File S2: Mussel sampling datasheet.

File S3: A primer on analysis methods.

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